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(54) Title: METHODS OF CONTROLLING MICROBIAL POLYESTER STRUCTURE

(57) Abstract

The addition of PEG to culture media of *Alcaligenes eutrophus* and *A. latus* resulted in the following: (1) the controlled decrease in polyhydroxyalkanoate (PHA) molecular weight, which decreases the melt viscosity and bioresorption time; (2) the modulation of the repeat unit composition of the PHA products containing 3-hydroxybutyrate, 3-hydroxyvalerate, and 4-hydroxybutyrate, which provides polymers with varied physical properties; (3) the alteration of PHA repeat unit sequence distribution so that complex polymeric mixtures are obtained in place of random copolymers; and (4) the formation of PHA-PEG diblock copolymers where the carboxylate terminus of PHA chains are covalently linked by an ester bond to PEG chain segments. This is an example of the cellular production of a naturally synthesized diblock copolymer. The invention features new diblock copolymers, copolyester blends, and methods of preparation.

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Methods of Controlling Microbial Polyester Structure

Statement as to Federally Sponsored Research

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National Science Foundation. The Government has certain
rights in the invention.

Background of the Invention

This invention relates to the control of the
10 structure of microbially-produced polyester compositions
such as polyhydroxyalkanoates.

Polyhydroxyalkanoates (PHAs) are a series of
optically active, thermoplastic, water insoluble
polyesters of alkanoic acids produced by various
15 microorganisms. Since natural microbial PHAs are
synthesized in aqueous media from renewable resources to
form biodegradable thermoplastics, this process for
polymer synthesis is an "environmentally friendly"
preparative route. The microbial synthesis also avoids
20 the use of organic solvents and toxic chemicals required
for the chemical synthesis of PHAs. Also, since these
microbial polyesters are biodegradable, they can be
disposed of as part of the biowaste fraction of municipal
solid waste.

25   The first member of the PHA family to be
identified was poly(3-hydroxybutyrate), also known as
"P3HB." See, e.g., Lemoigne, *Ann. Inst. Pasteur (Paris)*,
39:144, 1925; Lemoigne, *Bull. Soc. Chim. Biol.*, *8*:770,
1926; and Lemoigne, Ann. *Inst. Pasteur (Paris)*, *41*:148,
30 1927. A problem associated with P3HB is that melt-
crystallized and solution-cast films of P3HB show brittle
behavior which increases upon aging at room temperature.

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PHAs with improved physico-mechanical properties have been created by incorporating different structural repeat units into PHAs. Over 50 different structural repeat unit types have been incorporated into PHAs to
5 produce a large range of homo- and copolyesters. This structural diversity has been achieved by using different microbial production systems and by varying media carbon sources. These carbon sources are metabolized into hydroxyalkanoate repeat units having variable pendant
10 group structures and number of carbon atoms between ester linkages.

Selected examples include poly[3HB-3-hydroxyvalerate-co-3-hydroxyhexanoate] (also referred to as "P[3HB-3HV-co-3HH"), described in Brandl et al., *Int.*
15 *J. Biol. Macromol.*, 11:49 (1989); P[3HB-co-3HH] described in Shiotani et al., Japanese Pat. Appl. 93049 (1993), and Shimamura et al., *Macromolecules,* 27:878-880 (1994); and P3HV described in Steinbüchel et al., *Appl. Microbiol. Biotechnol.*, 39:443-449 (1993).
20 3-Hydroxyalkanoates that contain n-alkyl side groups with lengths generally from propyl to nonyl have also been produced, for example with functional side chain substituents such as phenyl and cyanophenoxy groups. A number of PHAs have also been reported that
25 contain 4-hydroxbutyrate (4HB) repeat units, such as P[3HB-co-4HB] described in Kunioka et al., *Polym. Commun.*, 29:174 (1988) and Kunioka et al., *Appl. Microbiol. Biotechnol.*, 30, 569 (1989), and terpolyesters of 3HB, 3HV, and 4HB, described in Kimura et al.,
30 *Biotechnol. Lett.*, 14(6):445-450 (1992). In addition, 3-hydroxy-propionate (3HP) and 4HB repeat units have been found in PHAs produced by the bacterium *Alcaligenes eutrophus* (see, Kunioka et al., *Polym. Commun.*, 29:174-76, 1988, and Nakamura et al., *Macromol. Reports,*
35 A28(Suppl.1):15, 1991).

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Control of composition for copolyesters of 3HB and
4HB is normally achieved by variation in the carbon
sources used or by alteration of other physiological
parameters such as the incubation time and nitrogen
5 concentration. For example, see, Nakamura et al.,
Macromolecules, 25, 4237-4241 (1992), and Doi, Y.,
Microbial Polyesters, VCH, New York (1990). In this way,
PHAs can be chemically tailored to exhibit the desired
physical-mechanical properties, crystallization rates,
10 optical clarity, rheological properties, and
biodegradation rates.

Thus, a variety of carbon source substrates have
been used to form novel PHAs. However, known microbial
synthesis methods provide no rational strategies to
15 control polymer molecular weight or end group structure
during the microbial polymerization.

Summary of the Invention

The invention is based on the discovery that when
polyethylene glycol (PEG) of a known molecular weight is
20 added to the culture medium of the bacterium *Alcaligenes
eutrophus* or *Alcaligenes latus*, the structure of the
resulting product can be controlled. For example, when
A. eutrophus is placed in 4.0% PEG-200 supplemented media
under polymer producing conditions, PEG-200 interacts
25 with enzyme systems involved in PHA biosynthesis to cause
dramatic product structural modulation. The cells
respond to the PEG external stimulus by accumulating
large quantities of oligomeric PEG that has a number
average molecular weight (M_n) closely resembling that of
30 the PEG added to cultivation media.

Specifically, addition of PEG-200 to culture media
resulted in the following: (1) the controlled decrease
in PHA molecular weight, which decreases the melt
viscosity and bioresorption time; (2) the modulation of

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the repeat unit composition of the PHA products containing 3HB, 3HV, and 4HB, which provides polymers with varied physical properties; (3) the alteration of PHA repeat unit sequence distribution so that complex

5 polymeric mixtures are obtained in place of random copolymers; and (4) the formation of PHA-PEG diblock copolymers where the carboxylate terminus of PHA chains are covalently linked by an ester bond to PEG chain segments. This is an example of the cellular production

10 of a naturally synthesized block copolymer.

In general, the invention features a method for producing a PHA having a controlled, e.g., decreased, molecular weight by culturing a PHA-producing microorganism, e.g., *Alcaligenes*, in a polymer production

15 medium under conditions that allow the microorganism to produce a PHA, and adding PEG to the polymer production medium in an amount sufficient for the microorganism to produce a PHA having a molecular weight that is decreased relative to the molecular weight of a PHA produced by the

20 same microorganism under the same growth conditions without PEG.

As used herein, a "polymer production medium" is used, e.g., for the second-stage fermentation, and includes a desired carbon source, but has a deficiency in

25 one or more nutrients, e.g., nitrogen, oxygen, sulfur, or phosphate, that induces the microorganism to produce PHAs. These media are well known in the fermentation arts.

When *A. eutrophus* is used, the PEG can be added to

30 the polymer production medium at a concentration of, e.g., 0.25 to 10.0 percent (weight/volume). When *A. latus* is used, the PEG can be added to the polymer production medium at a concentration of, e.g., up to 6.0 percent (weight/ volume).

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In another embodiment, the invention features a method for incorporating 3-hydoxyvalerate (3HV) repeat units into a PHA using a non-3HV carbon source, e.g., 4-hydroxybutyric acid or 4-hydroxybutyrate, by culturing a
5 PHA-producing microorganism in a polymer production medium containing a non-3HV carbon source under conditions that allow the microorganism to produce a PHA, and adding PEG to the polymer production medium in an amount sufficient for the microorganism to produce a PHA
10 comprising 3HV, e.g., at a concentration of from 1 to 4 percent (weight/volume).

The invention further features a method for producing a PHA comprising a copolyester blend of at least two component polymers wherein each component
15 polymer represents at least 30 percent by weight of the total blend, each component polymer is composed of at least 70 percent of a specific repeat unit structure, and the major repeat unit structure in each component polymer is different. This method is carried out by culturing a
20 PHA-producing microorganism in a polymer production medium containing a carbon source, e.g., 4-hydroxybutyrate, under conditions that allow the microorganism to produce a PHA, and adding PEG to the polymer production medium in an amount sufficient for the
25 microorganism to produce a PHA comprising a copolyester blend, e.g., at a concentration of 4 percent (weight/volume).

The invention also features a method for producing a polyhydroxyalkanoate-polyethylene glycol (PHA-PEG)
30 diblock copolymer in which the carboxyl terminus of a PHA chain segment is covalently linked by an ester bond to a PEG chain segment by culturing a PHA-producing microorganism in a polymer production medium under conditions that allow the microorganism to produce a PHA,
35 and adding PEG to the polymer production medium in an

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amount sufficient for the microorganism to produce a PHA-PEG diblock copolymer.

For example, the polymer production medium can include glucose as the carbon source, and the
5 microorganism can be *A. latus*. Then PEG can be added at a concentration of up to 6 percent (weight/volume). This method can be used to produce a PHA chain segment containing only P3HB repeat units. In addition, the polymer production medium can include 4-hydroxybutyric
10 acid as the carbon source, and the microorganism can be *A. eutrophus*. Then the PEG can be added at a concentration of, e.g., 4 percent (weight/volume). This method can be used to produce a diblock copolymer comprising a majority of 4HB repeat units.

15   In another embodiment, the invention features a method for increasing the 4-hydroxybutyrate (4HB) mol percent in a PHA by culturing a PHA-producing microorganism in a polymer production medium containing 4-hydroxybutyric acid as a carbon source under conditions
20 that allow the microorganism to produce a PHA, and adding PEG to the polymer production medium in an amount sufficient for the microorganism to produce a PHA of increased 4HB mol percent, e.g., 1 or 2 percent (weight/volume).

25   In another aspect, the invention features a PHA copolyester blend including first and second polymers each comprising at least 30 percent by weight of the blend, wherein the first polymer comprises at least 70 mol percent of a first repeat unit structure, the second
30 polymer comprises at least 70 mol percent of a second repeat unit structure, and wherein the first and second repeat unit structures are different. For example, the first repeat unit structure can be 3-hydroxybutyric acid, the first polymer can comprise at least 90 mol percent of
35 a first repeat unit structure, the second repeat unit

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structure can be 4-hydroxybutyrate, the second polymer
can comprise at least 80 mol percent of a second repeat
unit structure.

The invention also features a
5   polyhydroxyalkanoate-polyethylene glycol diblock (PHA-
PEG) copolymer including a first chain of PHA repeat
units and a second chain of PEG repeat units, wherein the
second chain of PEG repeat units is covalently bound via
an ester bond to a carboxy terminal end of the first
10   chain of PHA repeat units. In particular examples, the
first chain can be poly(3-hydroxybutyrate), and the
second chain can have an average of 5 PEG repeat units,
in which case the first chain can comprise an average of
220 PHA repeat units, or the first chain can comprise at
15   least 80 mol percent of 4-hydroxybutyrate, and the second
chain can have an average of 5 PEG repeat units, in which
case the first chain can have an average of 435 PHA
repeat units.

Unless otherwise defined, all technical and
20   scientific terms used herein have the same meaning as
commonly understood by one of ordinary skill in the art
to which this invention belongs. Although methods and
materials similar or equivalent to those described herein
can be used in the practice or testing of the present
25   invention, the preferred methods and materials are
described below. All publications, patent applications,
patents, and other references mentioned herein are
incorporated by reference. In addition, the materials,
methods, and examples are illustrative only and not
30   intended to be limiting.

Other features and advantages of the invention
will be apparent from the detailed description, and from
the claims.

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Brief Description of the Drawings

Fig. 1 is a 500 MHz ^{1}H NMR Spectrum of purified PHA (*A. eutrophus*, carbon source (C.S.) 4-hydroxybutyrate, 4% PEG-200).

5   Fig. 2 is an expansion of a two dimensional homonuclear (^{1}H) correlated (COSY) spectrum of PHA (*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200).

Figs. 3a to 3c are a series of gel permeation chromatography (GPC) traces of products formed (3a:*A.*

10 *eutrophus*, C.S. 4-hydroxybutyrate, 0% PEG-200, crude; 3b:*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200, crude; and 3c:*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200, one time precipitated).

Fig. 4 is a 125 MHz ^{13}C NMR spectrum of PHA (*A.*

15 *eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200).

Figs. 5a to 5c are a series of expanded 75 MHz ^{13}C NMR spectra for carbonyl resonances of PHA (5a:*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200; 5b:Acetone soluble (AS) fraction of sample 5a; and 5c:Acetone

20 insoluble (AIS) fraction of sample 5a).

Figs. 6a to 6c are a series of differential scanning calorimetry (DSC) thermograms of PHAs (First Heating)(6a:*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200; 6b:Acetone soluble fraction of sample 6a; and

25 6c:Acetone insoluble fraction of sample 6a).

Figs. 7a to 7c are a series of DSC thermograms of PHAs (Second Heating) (7a:*A. eutrophus*, C.S. 4-hydroxybutyrate, 4% PEG-200; 7b:Acetone soluble fraction of sample 7a; and 7c:Acetone insoluble fraction of sample

30 7a).

Fig. 8 is a graph showing the effects of PEG-200 media concentration on the number average molecular weights (M_n) of the resulting PHAs from *A. eutrophus* and *A. latus*.

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Detailed Description
Use of PEG to Control PHA Structure

PEG with M_n of about 200 g/mol (PEG-200) was added
in an amount up to 10% (w/v) to cultivations of *A.*
5 *eutrophus* and 6% to cultivations of *A. latus*, either
initially or during the polymer production stage, e.g.,
the second stage of a two stage fermentation, to study:
(1) the effect of PEG-200 on the conversion by *A.*
eutrophus and *A. latus* of the carbon source 4-
10 hydroxybutyrate (4HB) to polyester, (2) changes in
product molecular weight, and (3) the incorporation of
PEG chain segments that are covalently linked to
microbial polyester products.

Gel permeation chromatography (GPC) was used to
15 investigate product molecular weight averages and
dispersity. One- and two- dimensional ^{1}H nuclear
magnetic resonance (NMR) spectroscopy were used to study
the repeat unit composition and incorporation of PEG-200
in various product fractions. ^{13}C NMR spectroscopy was
20 used to analyze polymer repeat unit sequence
distribution. Fractionation by differential solubilities
in acetone was used to investigate product heterogeneity.
In addition, differential scanning calorimetry (DSC) was
used to obtain information on thermal transitions of
25 products and product fractions.

Polyethylene Glycol

PEG-200 (200 g/mol) used in these studies was
purchased from Aldrich. The PEG number average molecular
weight (M_n) was confirmed by using ^{1}H NMR end group
30 analysis and was found to be 194 g/mol.

Bacterial Preservation and Inoculum Preparation

Alcaligenes eutrophus (ATCC 17699) was used in
this study. This strain was first grown under aerobic
conditions as described in Ervine, Chap. 2 in
35 <u>Fermentation, A Practical Approach</u> (McNeil et al. (eds.),

IRL Press, 1990) at 30°C for 14 hours, the culture was then diluted with 2 parts of 20% glycerol and transferred into 1 mL cryogenic vials. The vial contents were frozen in a dry ice-ethanol bath and then stored in liquid

5 nitrogen. The cells contained in the vials were used as the inoculum for the two-stage fermentation reactions described below.

Alcaligenes latus (DMS 1122) was also used in the methods of the invention.

10   A. eutrophus Fermentation Conditions

100 mL Cultivations (Cultivation Condition A): A nutrient rich medium (100 mL, as described in Kunoika et al., Appl. Microbiol. Biotech., 30:569, 1989) was prepared, autoclaved to sterilize, and inoculated with

15 0.1 mL cells from a thawed cryovial. A. eutrophus was grown in 500 mL baffled Erlenmeyer flasks in a shaker incubator at 30°C, 250 RPM, for 24 hours. The cells were harvested by centrifugation (4°C, 8,000 rpm for 20 minutes) and washed with a sterile Na_2HPO_4-NaH_2PO_4 buffer

20 solution at pH 7.0. Typically, the cell dry weight of these first stage cultivations was 0.5 g/L. The washed cells were then transferred under aseptic conditions into 100 mL of a sterile filtered nitrogen-free medium which contained 1.51 g/L Na_2HPO_4, 2.65 g/L KH_2PO_4, 0.2 g/L

25 $MgSO_4$, 1.0 mL/L Microelement solution (Kunioka et al., Appl. Microbiol. Biotechnol., 30, 569, 1989), 4-hydroxybutyric acid (1.5 g) or fructose (1.5 g), and either 0, 1, 2, or 4% (wt/vol) PEG-200. Polymer production was then carried out by cultivation of A.

30 eutrophus in the above media using a 500 mL Erlenmeyer flask at 30°C, 250 RPM, for 48 hours. The cells were then separated by centrifugation, washed with about 10 mL of water per gram of wet cells, and lyophilized.

500 mL Cultivations (Cultivation Condition B):

35 Increased PHA from media amended with 4% PEG needed for

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fraction and subsequent analysis (see below) was
obtained as described above by the two-stage method but
using 2800 mL Erlenmeyer flasks and 500 mL cultivation
volumes.

5   A. *latus* Fermentation Conditions

A. *latus* was grown on 1.0% glucose (w/v) in a one-
stage fermentation where PEG-200 was added to the initial
growth media as described in Hiramitsu, et al.,
Biotechnol. Letters 15:461 (1993). It was anticipated
10 that under these conditions, the PHA formed by A. *latus*
would be P3HB. A. *latus* grew and divided initially in
the presence of up to 3% (w/v) PEG-200, but showed no
bacterial growth, and hence no PHA production at 4% PEG-
200. Increasing the media concentration of PEG-200 from
15 0 to 1% caused little change in the cell and polymer
yields. However, at a media concentration of 3% PEG-200,
the biomass and polymer yield productivity dropped
precipitously.

Polymer Isolation

20   The intracellular PHAs formed from each
Alcaligenes species were extracted from cells by stirring
a suspension of lyophilized cells (about 0.5 g) for 48
hours in chloroform (80 mL) at room temperature. The
insoluble cellular material was removed by filtration,
25 and the solvent was then evaporated to obtain what is
termed herein the "crude product." Precipitated products
were isolated by concentrating the chloroform crude
product solution to a total volume of ~4 mL and
precipitation of the polymer in 30 mL of methanol. The
30 resulting precipitate was washed with methanol and ether
and then dried *in vacuo*. Unless otherwise specified, the
isolated products were obtained using one
precipitation/washing cycle.

The PHA formed from 4-hydroxybutyrate in the
35 medium with 4% PEG (cultivation condition B, see above)

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and isolated by one precipitation/washing cycle was
dissolved in chloroform (0.1 g/mL). Acetone (10 volumes)
was slowly added to the chloroform solution. The white
cotton-like precipitate which resulted from acetone
5   addition was isolated by filtration giving the acetone
insoluble (AIS) fraction. The solvent was evaporated
from the acetone-chloroform solution which gave the
acetone soluble (AS) fraction. Removal of residual
solvents from the AS and AIS fractions was carried out in
10 a vacuum dessicator (10 mm Hg, 24 hours) and the samples
were then allowed to age for at least one week at ambient
temperature prior to carrying out thermal analyses.

Polymer Characterization

A UNITY-500 NMR Spectrometer was used for 1 and 2-
15 D proton NMR experiments described below. Proton (^{1}H)
NMR were recorded at 500 MHz. Chemical shifts in parts
per million (ppm) were reported downfield from 0.00 ppm
using tetramethylsilane (TMS) as an internal reference.
The experimental parameters were as follows: 0.5% w/v
20 polymer in chloroform-d, temperature 298°K, 2.4 μsec
(14°) pulse width, 3 second acquisition time, and 6,000
Hz spectral width. Carbon (^{13}C) NMR spectra were recorded
using a Varian XL-300 at 75.4 MHz and the following
parameters: 2.0% w/v polymer in chloroform-d, 298°K, 9.7
25 μsec pulse width, 1 second acquisition time and 2 second
pulse delay, 16502 spectral width, 33024 data points, and
14400-19600 accumulations. The observed ^{13}C NMR chemical
shifts in ppm were referenced relative to chloroform-d at
76.91 ppm. For the COSY experiment (0.5% w/v polymer in
30 chloroform-d) the data were collected in a 1024 x 256
data matrix and zero-filled to 1024 x 1024 using 8 scans
per increment, a 4260 Hz sweep width, and a 1.1 second
delay between transients. The data was processed using
sinebell weighting.

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The polymers produced by *A. latus* were analyzed by 1-D-^{1}H NMR (500 MHz) and COSY (500 MHz) spectroscopy. Spectra of PHA samples were recorded on a Varian XU-500 spectrometer. Parameters for the 1-D-^{1}H and COSY polymer

5 spectra were as follows. 1.0% (w/v) polymer in CDCl$_3$, temperature 298°K, 2.4 and 14.5 μsec pulse widths, 8000 and 2710 spectral widths, 3.0 and 0.189 second acquisition times, 0 and 1.0 second delay times, and 45 and 8 transients, respectively.

10 The molecular weights of polyesters were determined by GPC studies using a Waters HPLC system with 500-, 10^3-, 10^4-, and 10^5-Å Ultrastyragel columns placed in series. Chloroform (HPLC grade) was used as the eluent at a flow rate of 1.0 mL/min, sample

15 concentrations were typically 3 to 10 mg/mL and the injection volume was 100 μL. Detection was by refractive index (Waters Model 410). Polystyrene standards (Aldrich) with low polydispersities were used to generate a calibration curve from which product molecular weights

20 were determined with no further corrections.

All thermal characterizations were carried out using a DuPont 2910 differential scanning calorimetry (DSC) equipped with a TA 2000 data station, using between 5.0 to 6.0 mg of sample sealed in aluminum pans and a dry

25 nitrogen purge. The polymer samples were heated at a rate of 10 °C/min from room temperature to 200°C, rapidly quenched from the melt and then were analyzed during second heating scans from -80°C to 200°C. Data reported for the melting temperature(s), T$_m$, and enthalpy of

30 fusion(s), ΔH$_f$, were taken from the first heating scan. The reported glass transition temperature (T$_g$) values were the midpoint values measured during the second heating scans.

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EXAMPLES
Effect of PEG on PHA Repeat Unit Composition

Control of composition for copolyesters of 3HB and 4HB is normally achieved by variation in the carbon
5 sources used or by alteration of other physiological parameters such as the incubation time and nitrogen concentration. In this study, PEG-200 was added to *A. eutrophus* cultivations in concentrations up to 4% (w/v) during the second or polymer producing stage of the
10 fermentation where 4HB served as the carbon source. The mol fractions of repeat units for PHAs isolated by one precipitation/washing cycle were analyzed by ^{1}H NMR spectral integration of well resolved signal regions (see Fig. 1) as has been previously described, e.g., in
15 Nakamura et al., *Macromolecules*, 25:4237-4241 (1992).

When *A. eutrophus* is grown on 4HB without PEG added, the resulting PHA closely approximates a random copolyester of 3HB and 4HB repeat units of high molecular weight, with no 3HV or EG repeat units (see Table I,
20 below). Upon the addition of PEG-200 in culture conditions A, dramatic shifts in the repeat unit composition were achieved. Table I below shows the effects of PEG-200 on the production and compositions of microbial polyesters formed by *A. eutrophus* using 4HB as
25 carbon source. In particular, the mol% of 4HB in the product changed from 66% with 0% PEG-200 added, to 86% with 2% PEG-200 added. Upon further addition of PEG-200 from 2 to 4%, the mol% of 4HB decreased. Furthermore, the addition of PEG-200 resulted in products containing
30 low level incorporation of 3HV repeat units (see Table I).

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Table I

polym, %PEG,	cult cond	cell yield, g/L	polym. content of cells, %	PHA yield, g/L	repeat units found in PHA mol %				M_n, g/mol X 10^{-3}	M_w/M_n
					3HB	4HB	3HV	EG		
0	A	3.7	21	0.76	34	66	0	0	222.1	2.76
1	A	3.5	16	0.56	20	79	1.1	0	178.6	1.89
2	A	3.1	14	0.45	11	86	2.8	0.28	153.0	2.05
4	A	2.6	14	0.37	30	64	5.0	0.93	112.2	2.51
0	B	3.9	27	1.1	70	30	0	0	198.6	2.87
4	B	3.7	26	0.97	41	53	5.4	1.1	77.2	3.95
4-AS	B				13	84	2.1	1.6	37.4	2.52
4-AIS	B				95	3	2.0	0.1	130.0	3.42

In Table I, PEG was added to the cultivation
medium during the second, polymer producing stage. Non-
15 fractionated samples were obtained from one
precipitation/ washing cycle. As described in further
detail below, 4-AS and 4-AIS are the acetone soluble (57%
w/w) and insoluble (43%) fractions of the 4% PEG product
obtained using cultivation condition B. The cell yield
20 is the quantity of harvested cells after they were washed
with nanopure water and lyophilized. The polymeric
content of the cells is expressed as the percent of the
cellular dry weight which contains PHA. These values
were obtained gravimetrically from the isolated product
25 from chloroform extraction and one precipitation/washing
cycle. The PHA yield is the (cell yield) X (fraction of
the cellular dry weight which is PHA). The M_n and M_w/M_n
were determined by GPC. The cultivation conditions are
either condition A or B as indicated. This experiment
30 was repeated and the identical trends were observed.

As shown in Table I, small quantities of PEG-200
added to fermentation media caused important product

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As shown in Table I, small quantities of PEG-200
added to fermentation media caused important product
compositional changes. The addition of PEG to the media
increased both 4HB and 3HV contents, while decreasing the
5 3HB contents. It is known, e.g., as described in Doi et
al., _Microbial Polyesters_ (VCH Publishers, N.Y. 1990),
that by decreasing the mol percentage of 3HB while
increasing the relative contents of 4HB or 3HV in
polymers, products are formed that have a relatively
10 higher flexibility and elongation at break.

Formation of PHA-PEG Diblock Copolymers

The ^{1}H NMR spectrum of the PHA isolated by one
precipitation/washing cycle for a cultivation containing
4% PEG-200 (culture condition B, see Table I) is shown in
15 Fig. 1. Weak ^{1}H NMR signals at ~3.7 ppm were observed
that correspond to protons (a,c,d,e) of ethylene glycol
(EG) repeat units. In contrast, P(3HB-co-4HB) formed in
the absence of PEG (not shown) does not show any ^{1}H NMR
signals in the 3.6 to 3.8 ppm spectral region.
20   A COSY spectrum of this product was recorded and
the specific spectral regions of interest are shown in
Fig. 2. Three ^{1}H signals at 4.25, 4.35, and 4.46 ppm
were observed that have correlations (coupling between
neighboring ^{1}H nuclei) with signals at 3.70, 3.73 and
25 3.77 ppm, respectively. The signal at 4.25 ppm also has
a contribution from a satellite peak of protons _8_ (4.1
ppm) (in Fig. 1) due to ^{13}C-^{1}H coupling. Based on
chemical shift parameters documented for model compounds,
it is expected that esterification of a terminal PEG-CH$_2$-
30 OH will lead to a downfield shift from ~3.7 to ~4.25 ppm.
Considering these results and data, the signals in
the 3.68 to 3.80 and 4.20 to 4.50 ppm regions were
assigned to protons _a_ and _b_, respectively, of esterified
PEG chain segments. Correlation of the signals with

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peaks at 3.62 and 3.73 suggest that they are due to protons e and d of terminal free hydroxyl EG units (see Fig. 2). Assuming that the contribution of the overlapping signals in the 3.6 to 3.8 region can be
5 estimated by Bernoullian curve fitting, the area under peaks was measured by cutting and weighing. The integration results showed that the ratio of protons a + d to c to 2x e was 3:6:2. Using the ratio c to a + d and c to 2x e gives values of n (internal EG units of 2 and
10 3, respectively).

Thus, the average chain length of PEG segments in the diblock copolymer is between 4 and 5 which corresponds to molecular weights of ~180 and 220 g/mol, respectively. The above results are consistent with the
15 formation of PHA chains that are covalently linked at the carboxylate chain terminus to PEG chain segments, which indicates that PHA-PEG diblock copolymers were formed (see Figs. 1 and 2). Furthermore, the average PEG chain length in the product is almost identical to that which
20 was provided in the cultivation media.

Such PHA-PEG diblock copolymers include a long PHA chain segment (average of 430 4HB repeat units) that is covalently linked with an ester bond at its carboxy terminal end to a relatively short PEG chain segment
25 (average of 5 repeat units). These PHA-PEG diblock copolymers provide unique characteristics compared to PHA products currently available. For example, the diblock copolymers include terminal ethylene glycol (EG) hydroxyl functionalities that allow the formation of chemical
30 linkages with drugs, they have amphipathic characteristics, and they can be used in blends as compatibilizing agents.

The invention also provides a unique method to incorporate PEG into PHA formulations such that the PEG
35 will leach out of the PHA into an aqueous media at a much

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slower rate than formulations in which PHAs and PEG are
merely mixed together.

The following experiments were performed to
provide further evidence that ^{1}H NMR signals observed in
5 the 3.6-3.8 ppm region for one-time-precipitated products
were not due in part to residual PEG-200. P(3HB-co-30%
4HB) (produced by a cultivation of A. eutrophus with no
added PEG-200) and PEG-200 (286 and 218 mg, respectively)
were dissolved in chloroform and cast to form a film.
10 This film contained 43% by weight PEG-200, which exceeds
by a factor of ~2 times the quantity of PEG-200 found in
the corresponding crude product (non-precipitated-
solution extracted material, see discussion below). The
film was then purified by one precipitation/washing cycle
15 using identical conditions as was used for isolated
products. The resulting isolate contained 0 mol % PEG
based on ^{1}H NMR analysis. Therefore, no residual PEG
exists in the one-time-precipitated product.

In addition, PEG-200 (0.3% w/w) was mixed with a
20 PHA-PEG product (0.7% w/w) obtained after 3
precipitation/ washing cycles from 4% PEG amended
cultivations. Once again, after only one
precipitation/washing cycle, the relative signal
intensities of the 3.6-3.8 ppm signal region to PHA
25 protons was identical to that of the PHA-PEG product
prior to mixing with PEG-200. Moreover, repeated
precipitation/washing (up to three times) of one-time-
precipitated samples did not result in a change in the EG
mol percentage. Therefore, non-covalently linked PEG-200
30 is indeed removed efficiently from the isolated products
by one precipitation/washing cycle.

Formation of Copolyester Blends

PHAs isolated from A. eutrophus cultivations in
which 4-hydroxybutyric acid served as a carbon source and

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PEG-200 was not added to the media have sequence
distributions of 3HB and 4HB repeat units that are
approximately random. See, e.g., Nakamura et al.,
Macromolecules, 25:4237-4241, 1992.

5   A GPC trace of the extracted crude material from
A. eutrophus cultivations (culture condition B using 4-
hydroxybutyrate as the carbon source, see Table I)
containing 4% PEG was quite complex indicating that it is
a mixture or blend of polymers having very different

10 molecular weight averages (see Fig. 3b). The mixture has
unique physical and biological properties. In contrast,
the GPC trace of the crude polymer product obtained from
cultivation media without PEG-200 shows only a unimodal
peak (see Fig. 3a). Also, the GPC trace of the crude

15 product has a component peak with an elution volume which
corresponds exactly with that of PEG-200 (Fig. 3b, peak
at 200 g/mol). This is further evidence that PEG-200
does indeed accumulate in the cells, and that this occurs
without notable cellular selectivity as a function of PEG

20 chain length.

          Consistent with the studies above in which PEG-200
was mixed with PHAs and removed by one
precipitation/washing cycle, the GPC trace of the one-
time-precipitated product (Fig. 3c) shows no trace of

25 residual PEG-200, but still shows multiple component
peaks.

                Fractionation of PEG Cultivation Products
          The PHA product was fractionated based on its
solubility in acetone. Fractionation resulted in an

30 acetone soluble fraction (AS) representing 57% (w/w) of
the total product, and an acetone insoluble fraction
(AIS) representing 43% (w/w) of the total product. The
AS and AIS fractions had M_n (M_w/M_n) values of 37,400
(2.52) and 130,000 (3.42), respectively (see Table I).

35 The fact that the PHA product could be fractionated

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provides additional evidence that the product is a
mixture or blend of polyesters as opposed to a block
copolymer.

5   Analysis of the repeat unit composition of these
fractions by ^{1}H NMR spectral integration showed that the
mol fractions of 3HB, 4HB, 3HV, and EG repeat units are
13, 84, 2.1, 1.6 and 95, 3, 2, 0.1, respectively (see
Table I). Thus, the addition of PEG-200 to cultivation
media results in the formation of a new product that is a
10   blend of polyesters in contrast to the random copolymers
formed in the absence of PEG-200.

Also, the PEG chain segments are found primarily
in the AS high 4HB fraction (see Table I). This is
evidence that for *A. eutrophus*, linkages between PEG and
15   PHA segments occur primarily between 4HB and EG repeat
units.

Using a model in which it is assumed that PEG
segments are at all carboxyl terminal positions of PHA
chains, the M_n calculated molecular weight based on ^{1}H NMR
20   spectral integration is 24,000 g/mol, whereas the
experimentally determined value from GPC is 37,400 g/mol.
From this analysis, the results are consistent with this
model.

NMR Analysis of the Copolyester Blend
25 Fractionations

The effects of PEG-200 on the repeat unit sequence
distribution were studied using the ^{13}C NMR spectra for
the one time precipitated/washed products obtained from
fermentations with 0 and 4% PEG-200 (culture condition
30   B). The ^{13}C NMR spectrum for the latter product is shown
in Fig. 4 and expansions of the carbonyl regions of the
unfractionated product, acetone soluble (AS) fraction,
and acetone insoluble (AIS) fraction are shown in Figs.
5a, 5b, and 5c, respectively. The assignments of the
35 observed signals, including those in the carbonyl region

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which are sensitive to effects of repeat unit sequence distribution, were made as described in, e.g., Nakamura et al., *Macromolecules*, 25:4237-4241 (1992).

To simplify the repeat unit sequence analysis
5 below, the small contributions from 3HV and EG repeat units were neglected so that the products were assumed to consist of only 3HB and 4HB repeat units. The relative mol fractions of 3HB*-3HB (3*3), 3HB*-4HB (3*4), 4HB*-3HB (4*3) and 4HB*-4HB (4*4) dyads (see Figs. 5a to 5c) were
10 determined by spectrometer integration and are given in Table II below, which shows the experimental and calculated comonomer dyad fractions for PHAs and product fractions formed in cultivations with and without PEG-200.

15 Experimental values were compared to those calculated assuming a Bernoullian or random statistical process for microbial catalyzed copolymerization using the following relationships (equations 1-3) where F_3 is the mole fraction of 3HB units in the polymer as
20 described, e.g., in Doi et al., *Macromolecules*, 21:2722 (1988):

$$[3*3] = F_3{}^2 \qquad\qquad (1)$$
$$[3*4] = [4*3] = F_3(1-F_3) \qquad\qquad (2)$$
$$[4*4] = (1-F_3)^2 \qquad\qquad (3)$$

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Table II

polym	Dyad Sequence							
% PEG,	3HB-3HB		3HB-4HB		4HB-3HB		4HB-4HB	
	exp	(calcd)	exp	(calcd)	exp	(calcd)	exp	(calcd)
0	0.60	(0.49)	0.14	(0.21)	0.10	(0.21)	0.16	(0.096)
4	0.57	(0.32)	0	(0.25)	0	(0.25)	0.43	(0.18)
4-AS	0.07	(0.03)	0.14	(0.15)	0.14	(0.15)	0.66	(0.67)
4-AIS	1.0	(0.85)	0	(0.07)	0	(0.07)	0	(0.01)

In Table II, each column shows the experimental
values (exp), determined by measuring the relative peak
areas for the carbonyl carbon ^{13}C NMR signals assigned
(see Figs. 5a to 5c) to the four dyad sequences, and the
calculated value (calcd), determined from equations 1 to
3, assuming a Bernoullian or random statistical process
and that the contribution of 3HV and EG repeat units can
be neglected. In the first column, the indicated percent
PEG was added to the cultivation medium during the second
or polymer producing stage. The non-fractionated sample
was obtained from one precipitation/washing cycle from a
cultivation carried out using 500 mL of media in a 2.8 L
shake flask. The AS and AIS fractions of the 4% PEG
product represent 57% and 43% (w/w) of the product,
respectively.

Table II shows that the PHA produced with 0% PEG
approximates a random copolyester. In contrast, the
addition of 4% PEG to cultivations resulted in a novel
product that has predominantly 3HB*-3HB and 4HB*-4HB
dyads (see also, Table I rows 4-AS and 4-AIS, and Fig.
5a). Thus, the addition of PEG to the growth medium
provides a new microbial polymerization process in which
polymer blends are made directly by a single fermentation
reaction. Therefore, this new process is much more

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efficient than prior methods to produce blends of polymers in which one component polymer has a high (greater than 70%, and preferably greater than 90%) 3HB content, and the other component polymer has a high

5   (greater than 70%, and preferably greater than 80%) 4HB content. Furthermore, each of the two component polymers represents at least 30% of the total weight of the blend.

Thermal Analysis of the Copolyester Blend Fractionations

10   Table III, below, shows the results of thermal analysis obtained by DSC measurements at a scanning rate of 10°C/min. The percent PEG added to the cultivation medium was added during the second or polymer producing stage. The non-fractionated samples were obtained from

15 one precipitation/washing cycle. Again, 4-AS and 4-AIS are the acetone soluble (57% w/w) and insoluble (43%) fractions of the 4% PEG sample. In Table III, T_g represents glass transition temperatures taken as the midpoint of the heat capacity change and measured during

20 the second heating scan after rapidly quenching by liquid nitrogen at -70°C from the melt. T_m represents the peak melting temperatures for each endothermic melting transition determined during the first heating scan. ΔH_f (cal/g) represents the heat of fusion value measured for

25 each melting endothermic transition. Cultivations were carried out using 500 mL of media in a 2.8 L shake flask (culture conditions B).

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<u>Table III</u>

polym, (% PEG w/v)	T_g (°C)	T_m (°C)	ΔH_f (cal/g)
0	3 -15	165	9.9
4	-45 -29	55 170	5.0 11.6
4-AS	-42 -15	50	8.8
4-AIS	2.8	172	19.0

As shown in Table III, the DSC thermograms of the 4% PEG product during a first heating scan showed two distinct T_m values at 55° and 170°C (see also Fig. 6a) which closely approximate reported T_m values for P3HB and P4HB (177° and 54° C, respectively). The DSC thermogram of this product recorded during a second heating scan after rapidly quenching from the melt showed T_g values at -45° and -29°C (see also Fig. 7a). The T_g at -45°C closely approximates that reported for P4HB (-50°C) while the T_g at -29°C is intermediate to those reported for P3HB (~4°C) and P4HB. This indicates the formation of a copolymer blend of predominantly P3HB and P4HB. The observed T_g at -29°C may result from the formation of a small product fraction that consists of random 3HB/4HB copolyester chains having the corresponding T_g value. In contrast, the product obtained from cultivations with no added PEG had T_m and T_g values of 165 and 3°C, respectively, which is consistent with the formation of a random copolyester.

^{13}C NMR and DSC measurements of the AS and AIS fractions were made to further characterize the individual component polymers of the copolyester blend formed in 4% PEG amended media. Expansions of the ^{13}C NMR carbonyl spectral regions for these fractions are shown in Figs. 5b and 5c, respectively. DSC thermograms of the

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first and second heating scans are shown in Figs. 6a to
6c and 7a to 7c, respectively. If the T_g, T_m, and ΔH_f
values for solution precipitated P3HB are taken as 4°C,
20.8 cal/g, and 177°C, respectively, comparison of these
5 data to those obtained for the AIS fraction (see Table
III) indicates that this fraction contains primarily P3HB
homopolymer, as opposed to a random copolyester such as
P(3HB-co-6 mol% 4HB) that has been shown to have T_m and
ΔH_f values of 162°C and 13.5 cal/g, respectively
10 (Nakamura et al., *Macromolecules*, 25:4237-4241, 1992).
This is further supported by the ^{13}C NMR spectrum of the
AIS fraction which shows only 3HB*-3HB dyads (see Fig.
5c).

The dyad sequence distribution of the AS fraction
15 determined experimentally (see Fig. 5b), and calculated
using equations 1 to 3, above, suggests that the product
formed approximates that of a high 4HB content random
copolyester (see Table II). Further study of this
fraction by DSC indicated product heterogeneity.
20 Specifically, the AS fraction had multiple Tg (-15,-42°C)
transitions and a broad melting region (see Table III,
Figs. 6b and 6b). A comparison of the thermal
transitions of this product fraction with those
previously reported for 3HB/4HB random copolyesters
25 (Nakamura, 1992, supra) indicates that the AS fraction is
composed of P(3HB-co-90% 4HB) and P(3HB-co-28% 4HB)
random copolyesters (T_g values of -44 and -15°C,
respectively)(Nakamura, 1992, supra).

This analysis, assuming that the components are
30 immiscible, indicates that the AS fraction is a mixture
of random copolyesters with relatively high and low 4HB
contents (~90-94 and ~30 mol%, respectively) with weight
fractions of ~86 and 14%, respectively. Thus, it appears
that the unfractionated product from media containing 4%
35 PEG is indeed complex as was originally indicated by the

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GPC trace (see Figs. 3b and 3c, above), and is composed
of at least three different component polymers of
different repeat unit composition.

Effect of PEG on PHA Molecular Weight and Yield
5   Results of Studies on *A. eutrophus* (C.S. 4-
Hydroxybutyric Acid)

         Table I also depicts the effects of PEG-200 on
volumetric yield and product molecular weight for the
series of fermentations of *A. eutrophus* carried out under
10 culture conditions A. The volumetric yield of the PHAs
continued to decrease with increased PEG media
concentration so that for 2 and 4% PEG-200 addition the
yields were approximately 59% and 49%, respectively, of
that for PEG deficient media. The M_n and M_w/M_n values
15 measured by GPC of the products formed from cultivations
with 0, 1, 2, and 4% PEG are also shown in Table I. The
GPC traces of these products were unimodal. An increase
in the PEG concentration from 0 to 4 percent resulted in
a decrease in product molecular weight (M_n) from 222,100
20 g/mol to 112,200 g/mol, e.g., about 50 percent. Thus,
PEG can be used to form PHAs that contain 4HB repeat
units and have reduced molecular weights compared to PHAs
produced without PEG. A decrease in molecular weight
affects the polymer characteristics, e.g., decreases the
25 melt viscosity, and is useful to form sustained release
compositions and biomaterials that require relatively
shorter bioresorption times.

         Comparison of PEG Effects on P3HB Molecular Weight
         in *A. latus* and *A. eutrophus*
30   PEG-200 was added to the fermentation media of
each organism either initially, or at the beginning of
the second stage of a two stage fermentation. Table IV
shows the effects of PEG-200 on bacterial growth, polymer
production, and polymer composition from 1 and 2 stage

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cultivations of *A. latus* grown on glucose (designated by the letter L), and 2 stage cultivations of *A. eutrophus* grown on fructose (designated by the letter E). In Table IV, R-CDW is "residual cell dry weight" which corresponds 5 to the non-polymer weight of the cells which may be considered the residual biomass.

Table IV

Product	Initial PEG-200 conc. (%), and incubation time (hr)	PEG-200 supplement final conc. (%), incubation time (hr)	Cell Yield (g/L)	Polymer Yield (g/L)	Cellular Productivity (mg/mg R-CDW)[a]	Compositions (mol%) [3HB (EG)]
Alcaligenes latus DSM 1122						
1 Stage Fermentation						
L-1	0 (48)		1.9	2.8	1.5	100(0)
L-2	1 (48)		1.8	2.7	1.5	98.2(1.8)
L-3	2 (48)		1.6	2.1	1.3	97.7 (2.3)
L-4	3 (48)		0.9	0.3	0.3	97.8 (2.2)
2 Stage Fermentation						
L-5	2(24)	4(24)	1.6	1.4	0.9	96.3 (3.7)
L-6	2(24)	5(24)	1.7	1.3	0.8	96.4 (3.6)
L-7	2(24)	6(24)	1.9	0.8	0.4	96.4 (3.6)
Alcaligenes eutrophus ATCC 17699						
2 Stage Fermentation						
E-1	0(24)	0(48)	3.3	3.0	0.9	100(0)
E-2	0(24)	0.25(48)	3.0	2.9	1.0	100(0)
E-3	0(24)	0.5 (48)	2.8	2.9	1.0	100(0)
E-4	0(24)	1(48)	2.7	2.7	1.0	100(0)
E-5	0(24)	1.5(48)	2.6	2.5	1.0	100(0)
E-6	0(24)	2(48)	2.9	2.4	0.8	100(0)
E-7	0(24)	5(48)	1.7	1.7	1.0	100(0)
E-8	0(24)	10(48)	2.3	0.3	0.1	100(0)

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Results in *A. latus*

As shown in Table IV, increasing the media
concentration of PEG-200 from 0 to 1% caused little
change in the cell and polymer yields for *A. latus*.
5 However, at a media concentration of 3% PEG-200, the
biomass and polymer yield productivity dropped
precipitously. These results are likely due to the
increased osmotic stress caused by increasing media PEG-
200 concentrations.
10   Further, the cellular productivity of *A. latus* was
constant between 0 and 2% PEG-200 concentrations, but at
3% PEG-200, cellular productivity was reduced by 80% to
0.3 mg/mg R-CDW. This reduction indicated that above 2%
PEG 200, the decrease in product yield was due not only
15 to poorer cell yields, but was also the result of a less
efficient production system.
       The molecular weights of the isolated polymer
products formed by *A. latus* were analyzed by gel
permeation chromatography (GPC). Unless otherwise
20 specified, the isolated products were obtained using 2
precipitation/ washing cycles.
       The addition of only 1% PEG-200 to *A. latus*
cultivation media resulted in a decrease in the M_n by 85%
from 238,000 g/mol to about 35,000 g/mol (see Fig. 8).
25 Further increases in the media PEG-200 concentration from
1 to 3% resulted in little to no further molecular weight
change. Thus, low PEG-200 media concentrations (1%) can
be used to modify product molecular weight.
       For two-stage cultivations of *A. latus*, polymers
30 were formed in production medium containing up to 6% PEG-
200, where 2, 3, and 4% PEG were added to cultivations
containing 2% PEG-200 after a first-stage 24 hour
cultivation. This two-stage approach resulted in
polymers with M_n values as low as 19,000 g/mol.
35 Furthermore, products L-2 to L-7, when analyzed by 1- and

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2-D proton NMR as described above for products derived
from 4-hydroxybutyric acid, were shown to contain diblock
copolymers of a P3HB chain segment covalently linked at
its carboxyl terminal end to a PEG chain segment.

5   Results in A. eutrophus

         For comparative purposes, a series of two-stage
cultivations were carried out using A. eutrophus as the
microbial production system where variable quantities of
PEG-200 were added to cultivation media (see Table IV).
10 Cellular growth and polymer production for all A.
eutrophus cultures were carried out using a two-stage
batch culture process as described above for cultivation
conditions A, using fructose as the carbon source for the
second stage cultivations.

15   A. eutrophus showed only small decreases in cell
and product yield with the addition of up to 2% PEG-200.
Also, the cellular productivity calculated using the non-
polymer or residual cell dry weight (R-CDW, see Table IV)
remained almost unchanged (~1.0 mg/mg R-CDW) for media
20 containing up to 5% PEG-200, but decreased to 0.13 mg/mg
non-polymer CDW when the PEG-200 media concentration was
increased to 10%. Thus, A. eutrophus showed an excellent
tolerance to the osmotic stress imposed by the solute
PEG-200.

25   Fig. 8 shows that the molecular weight of product
polyesters was decreased by increasing the media PEG-200
concentration. In fact, there was a regular decrease in
product molecular weight as the PEG-200 concentration was
increased from 0 to 1% (M_n values of 650,000 g/mol and
30 104,000 g/mol, respectively). As was observed for A.
latus, further increases in the media PEG-200
concentration from 1 to 5% resulted in substantially less
molecular weight reduction per added PEG increment (see
Fig. 8). Thus, sensitive control of product molecular
35 weight was achieved by variation of the media PEG-200

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concentration from 0 to 1% for both *A. latus* and *A. eutrophus*.

USE

The methods described above allow the modification
of microbial polyester products by simply changing the
concentration of PEG added to the cultivation medium. By
controlling the amount of PEG added, one can control the
molecular weight, repeat unit composition and
distribution, and produce specified copolyester blends as
opposed to random copolyester chains.

In particular, such diblock copolymers and
copolyester blends of the invention can be used to make
biodegradable plastic articles and coatings, e.g., for
paper, that are manufactured by standard thermal
processing methods. These new copolyesters can also be
used for microencapsulation, e.g., of cells or drugs, to
produce cell growth matrices, and to produce biomedical
materials such as sutures, implants, and drug delivery
vehicles.

Other Embodiments

As described in Shi et al., *Polymer Preprints, Am.
Chem Soc.*, 36(1):430-432 (April 1995), *A. eutrophus* grown
on fructose resulted in the formation of P3HB with a high
M_n. When PEG-200 was added to the culture media, the
P3HB M_n decreased significantly. For example, the
addition of 0.2% PEG decreased P3HB M_n by a factor of 2.
Further increases in PEG concentration up to 10% resulted
in decreased P3HB molecular weight by a factor of about
10.

Copolyester blends including component polymers
having repeat unit structures other than 3HB and 4HB can
also be made according to the invention. For example,
component polymer repeat unit structures of 3-

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hydroxyvalerate, 3-hydroxyhexanoate, 3-hydroxy-4-
pentenoate, and 3-hydroxypropionate, can be prepared
using the corresponding acids, e.g., 3-hydroxyvaleric
acid, 3-hydroxyhexanoic acid, 3-hydroxy-4-pentenoic acid,
5 and 3-hydroxypropionic acid, as carbon sources.

In addition, other carbon sources can be used to
form 3HB and 4HB repeat unit structures. For example,
1,4-butane diol and 1,6-hexane diol can be used as carbon
sources to produce 4HB repeat unit structures. Thus, the
10 addition of PEG to polymer production media containing
these carbon sources will also cause an increase in the
4HB mol percentage compared to fermentation without the
added PEG.

It is to be understood that while the invention
15 has been described in conjunction with the detailed
description thereof, that the foregoing description is
intended to illustrate and not limit the scope of the
invention, which is defined by the scope of the appended
claims. Other aspects, advantages, and modifications are
20 within the scope of the following claims.

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Claims

1. A method for producing a polyhydroxyalkanoate (PHA) having a controlled molecular weight, said method comprising

5   culturing a PHA-producing microorganism in a polymer production medium under conditions that allow the microorganism to produce a PHA, and

         adding polyethylene glycol (PEG) to the polymer production medium in an amount sufficient for the

10 microorganism to produce a PHA having a molecular weight that is decreased relative to the molecular weight of a PHA produced by the same microorganism under the same growth conditions without PEG.

2. A method of claim 1, wherein the PHA-producing

15 microorganism is an *Alcaligenes* bacterium.

3. A method of claim 2, wherein the PHA-producing microorganism is *Alcaligenes eutrophus*, and wherein PEG is added to the polymer production medium at a concentration of 0.25 to 10.0 percent (weight/volume).

20   4. A method of claim 2, wherein the PHA-producing microorganism is *Alcaligenes latus*, and wherein PEG is added to the polymer production medium at a concentration of up to 6.0 percent (weight/volume).

         5. A method for incorporating 3-hydoxyvalerate

25 (3HV) repeat units into a polyhydroxyalkanoate (PHA) using a non-3HV carbon source, said method comprising

         culturing a PHA-producing microorganism in a polymer production medium containing a non-3HV carbon source under conditions that allow the microorganism to

30 produce a PHA, and

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adding polyethylene glycol (PEG) to the polymer
production medium in an amount sufficient for the
microorganism to produce a PHA comprising 3HV.

6. A method of claim 5, wherein the non-3HV
5 carbon source is 4-hydroxybutyric acid or 4-
hydroxybutyrate.

7. A method of claim 5, wherein PEG is added to
the polymer production medium at a concentration of from
1 to 4 percent (weight/volume).

10   8. A method for producing a polyhydroxyalkanoate
(PHA) comprising a copolyester blend of at least two
component polymers wherein each polymer represents at
least 30 percent by weight of the total blend, each
component polymer is composed of at least 70 percent of a
15 specific repeat unit structure, and the major repeat unit
structure in each component polymer is different, said
method comprising
culturing a PHA-producing microorganism in a
polymer production medium containing a carbon source
20 under conditions that allow the microorganism to produce
a PHA, and
adding polyethylene glycol (PEG) to the polymer
production medium in an amount sufficient for the
microorganism to produce a PHA comprising a copolyester
25 blend.

9. A method of claim 8, wherein PEG is added at a
concentration of 4 percent (weight/volume).

10. A method of claim 8, wherein the carbon
source is 4-hydroxybutyrate.

11. A method for producing a polyhydroxyalkanoate-polyethylene glycol (PHA-PEG) diblock copolymer in which the carboxyl terminus of a PHA chain segment is covalently linked by an ester bond to a
5 PEG chain segment, said method comprising

culturing a PHA-producing microorganism in a polymer production medium under conditions that allow the microorganism to produce a PHA, and

adding polyethylene glycol (PEG) to the polymer
10 production medium in an amount sufficient for the microorganism to produce a PHA-PEG diblock copolymer.

12. A method of claim 11, wherein the polymer production medium comprises glucose as the carbon source, and wherein the microorganism is *Alcaligenes latus*.

15   13. A method of claim 12, wherein PEG is added at a concentration of up to 6 percent (weight/volume).

14. A method of claim 12, wherein the PHA chain segment contains only P3HB repeat units.

15. A method of claim 11, wherein the polymer
20 production medium comprises 4-hydroxybutyric acid as the carbon source, and wherein the microorganism is *Alcaligenes eutrophus*.

16. A method of claim 15, wherein PEG is added at a concentration of 4 percent (weight/volume).

25   ,17. A method of claim 15, wherein the diblock copolymer comprises a majority of 4HB repeat units.

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18.   A method for increasing the 4-hydroxybutyrate
(4HB) mol percent in a polyhydroxyalkanoate (PHA), said
method comprising
        culturing a PHA-producing microorganism in a
5   polymer production medium containing 4-hydroxybutyric
acid as a carbon source under conditions that allow the
microorganism to produce a PHA, and
        adding polyethylene glycol (PEG) to the polymer
production medium in an amount sufficient for the
10   microorganism to produce a PHA of increased 4HB mol
percent.

        19.   A method of claim 18, wherein PEG is added to
the polymer production medium at a concentration of 1
percent (weight/volume).

15   20.   A method of claim 18, wherein PEG is added to
the polymer production medium at a concentration of 2
percent (weight/volume).

        21.   A polyhydroxyalkanoate (PHA) copolyester
blend comprising first and second polymers each
20   comprising at least 30 percent by weight of said blend,
wherein said first polymer comprises at least 70 mol
percent of a first repeat unit structure, said second
polymer comprises at least 70 mol percent of a second
repeat unit structure, and wherein said first and second
25   repeat unit structures are different.

        22.   A copolyester blend of claim 21, wherein said
first repeat unit structure is 3-hydroxybutyric acid.

        23.   A copolyester blend of claim 21, wherein said
first polymer comprises at least 90 mol percent of a
30   first repeat unit structure.

24.   A copolyester blend of claim 21, wherein said second repeat unit structure is 4-hydroxybutyrate.

25.   A copolyester blend of claim 21, wherein said second polymer comprises at least 80 mol percent of a
5 second repeat unit structure.

26.   A polyhydroxyalkanoate-polyethylene glycol diblock (PHA-PEG) copolymer comprising a first chain of PHA repeat units and a second chain of PEG repeat units, wherein the second chain of PEG repeat units is
10 covalently bound via an ester bond to a carboxy terminal end of the first chain of PHA repeat units.

27.   A PHA-PEG diblock copolymer of claim 26, wherein said first chain comprises poly-3-hydroxybutyrate, and said second chain comprises an
15 average of 5 PEG repeat units.

28.   A PHA-PEG diblock copolymer of claim 27, wherein said first chain comprises an average of 220 PHA repeat units.

29.   A PHA-PEG diblock copolymer of claim 26,
20 wherein said first chain comprises at least 80 mol percent of 4-hydroxybutyrate, and said second chain comprises an average of 5 PEG repeat units.

30.   A PHA-PEG diblock copolymer of claim 29, wherein said first chain comprises an average of 435 PHA
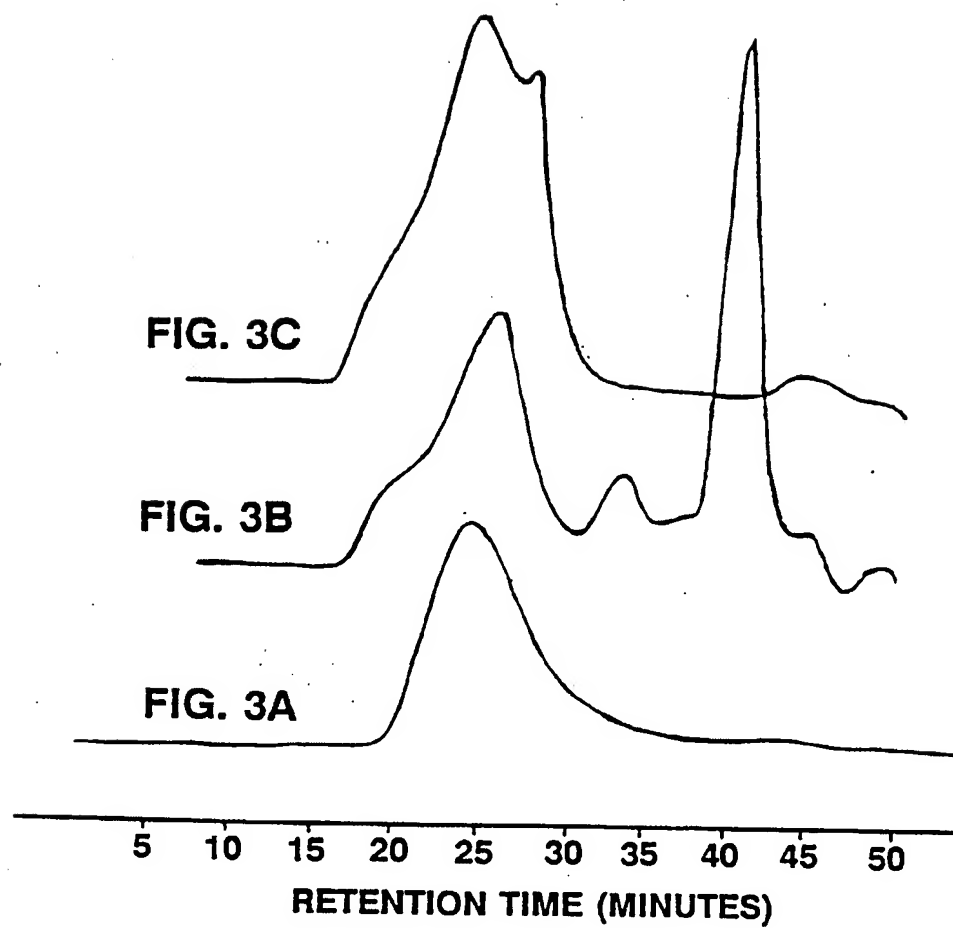25 repeat units.
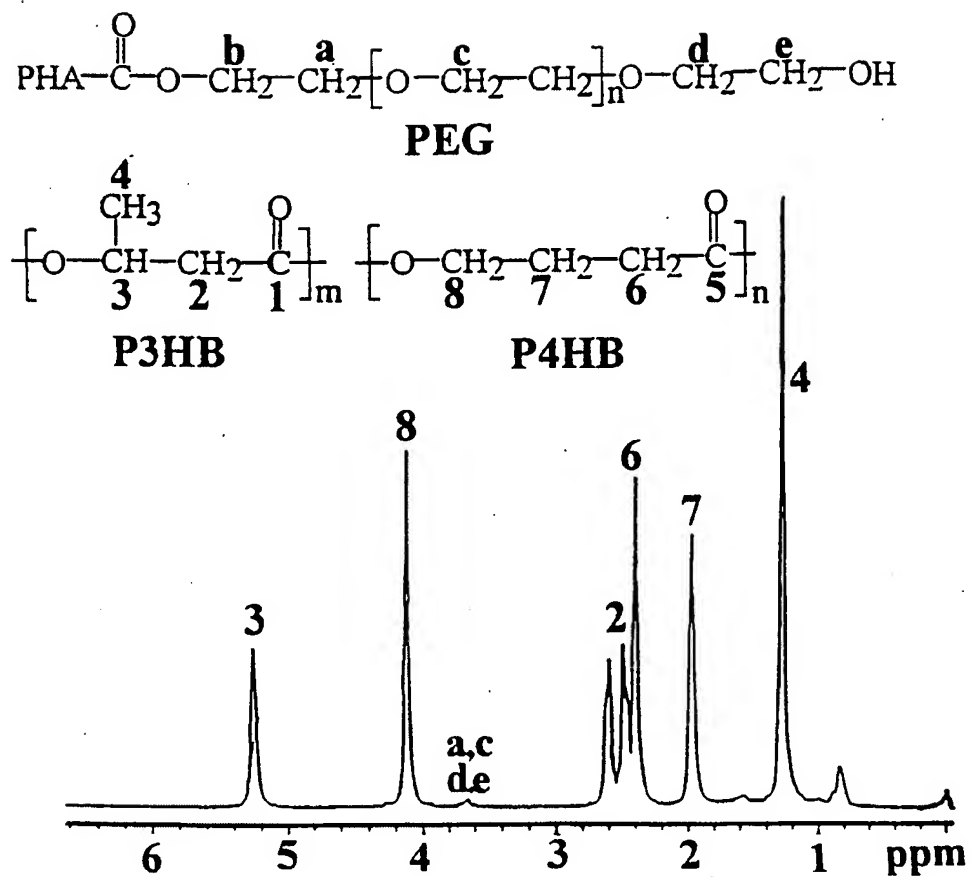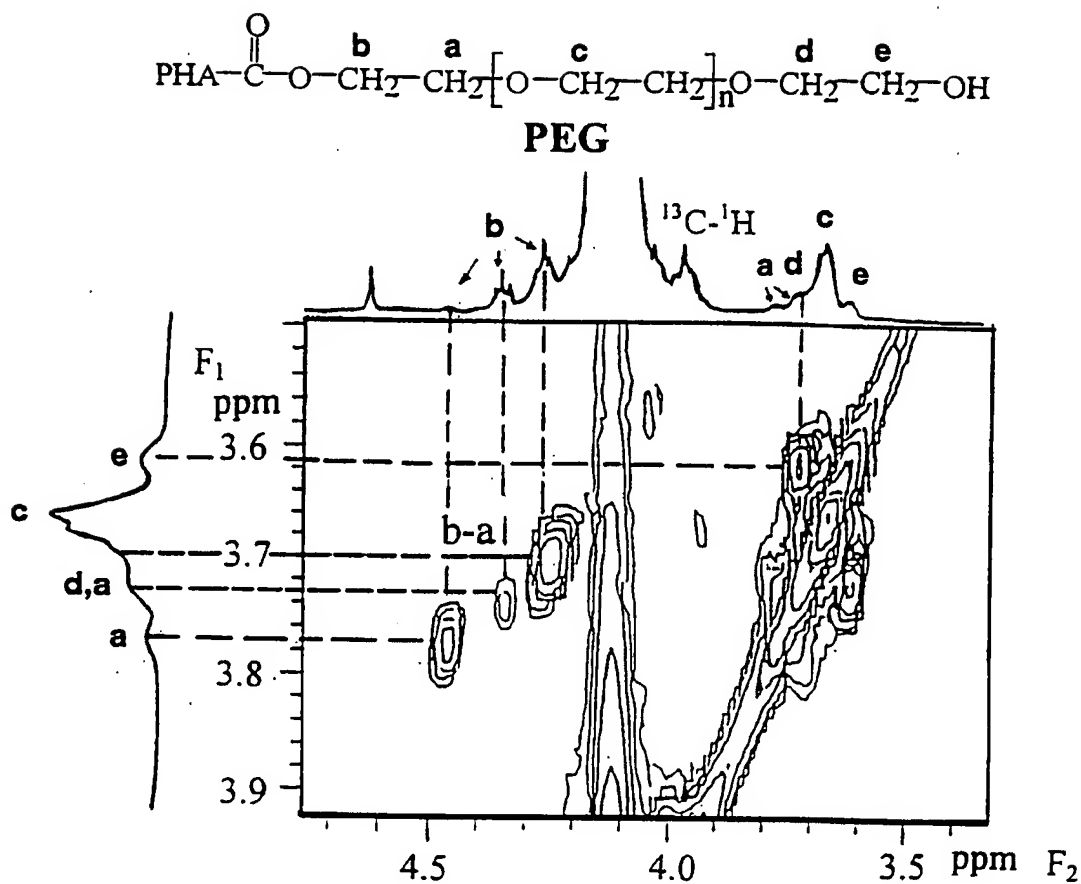
FIG. 1

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FIG. 2

7/3/07, EAST Version: 2.1.0.14

FIG. 3C

FIG. 3B

FIG. 3A

5   10   15   20   25   30   35   40   45   50

RETENTION TIME (MINUTES)

7/3/07, EAST Version: 2.1.0.14
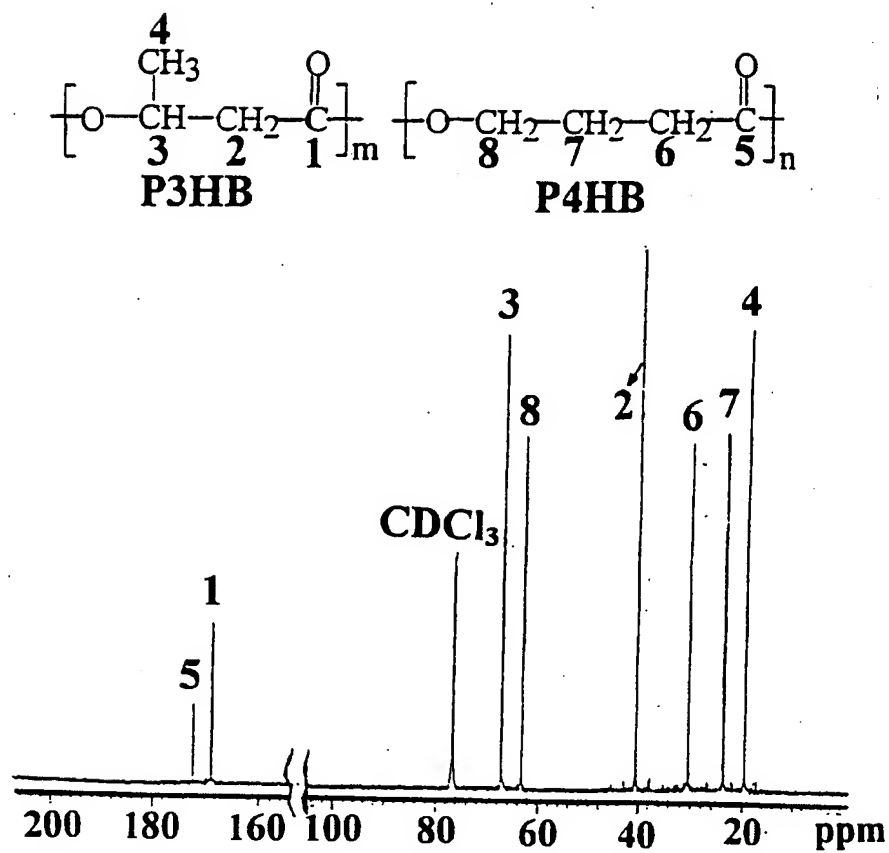
FIG. 4
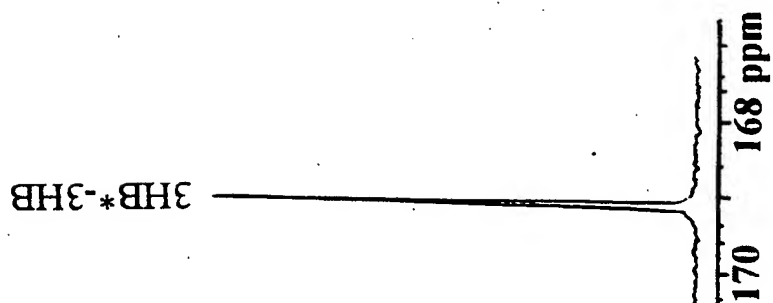
FIG. 5C
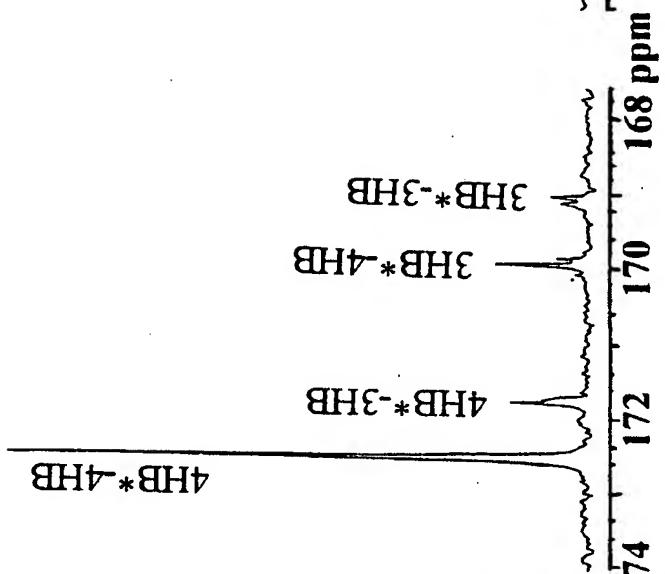
FIG. 5B
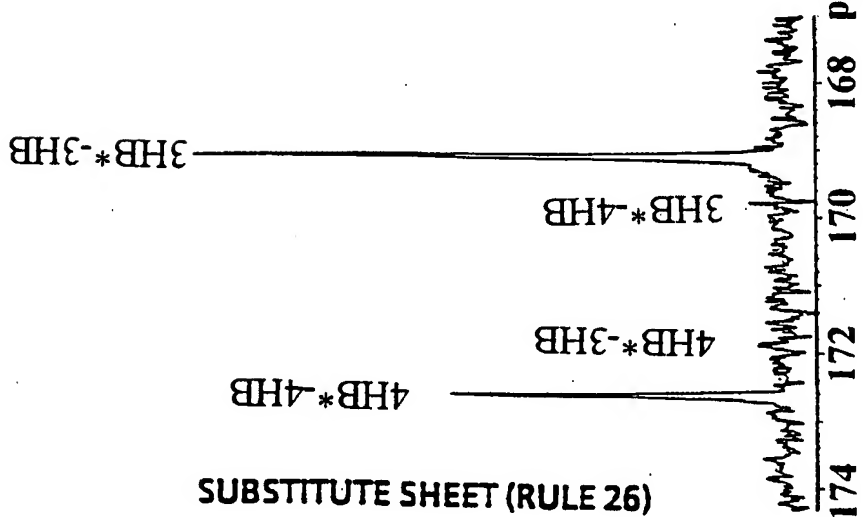
FIG. 5A

SUBSTITUTE SHEET (RULE 26)

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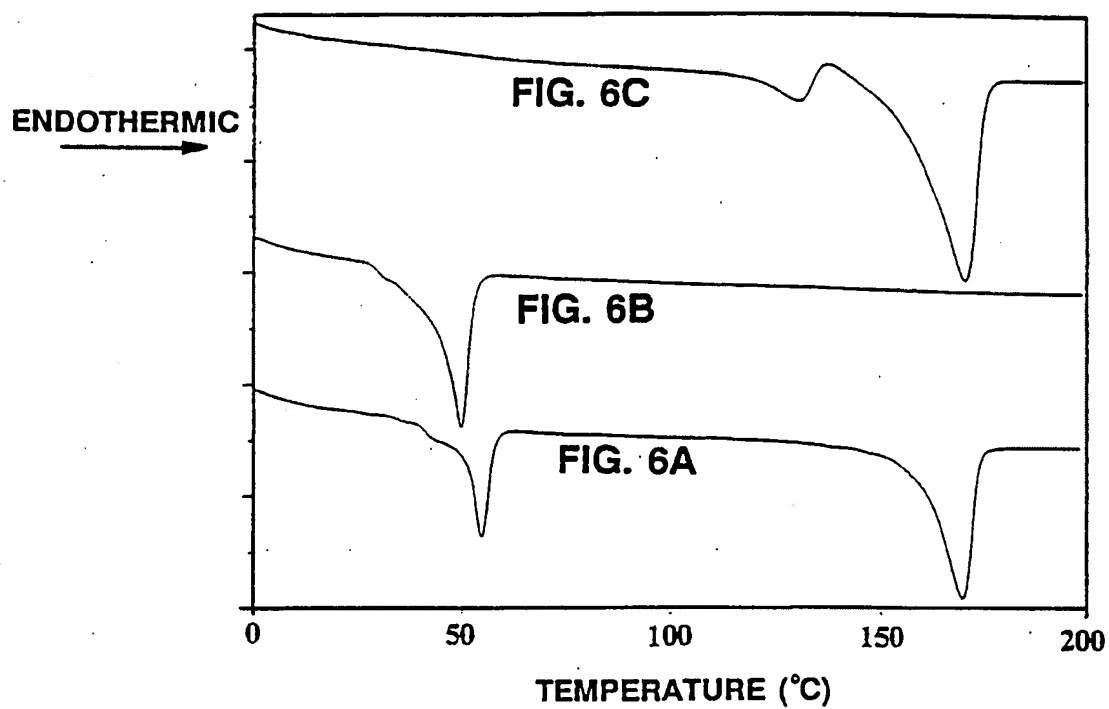
ENDOTHERMIC

FIG. 6C

FIG. 6B

FIG. 6A

TEMPERATURE (°C)

7/3/07, EAST Version: 2.1.0.14

FIG. 7A

ENDOTHERMIC

FIG. 7B

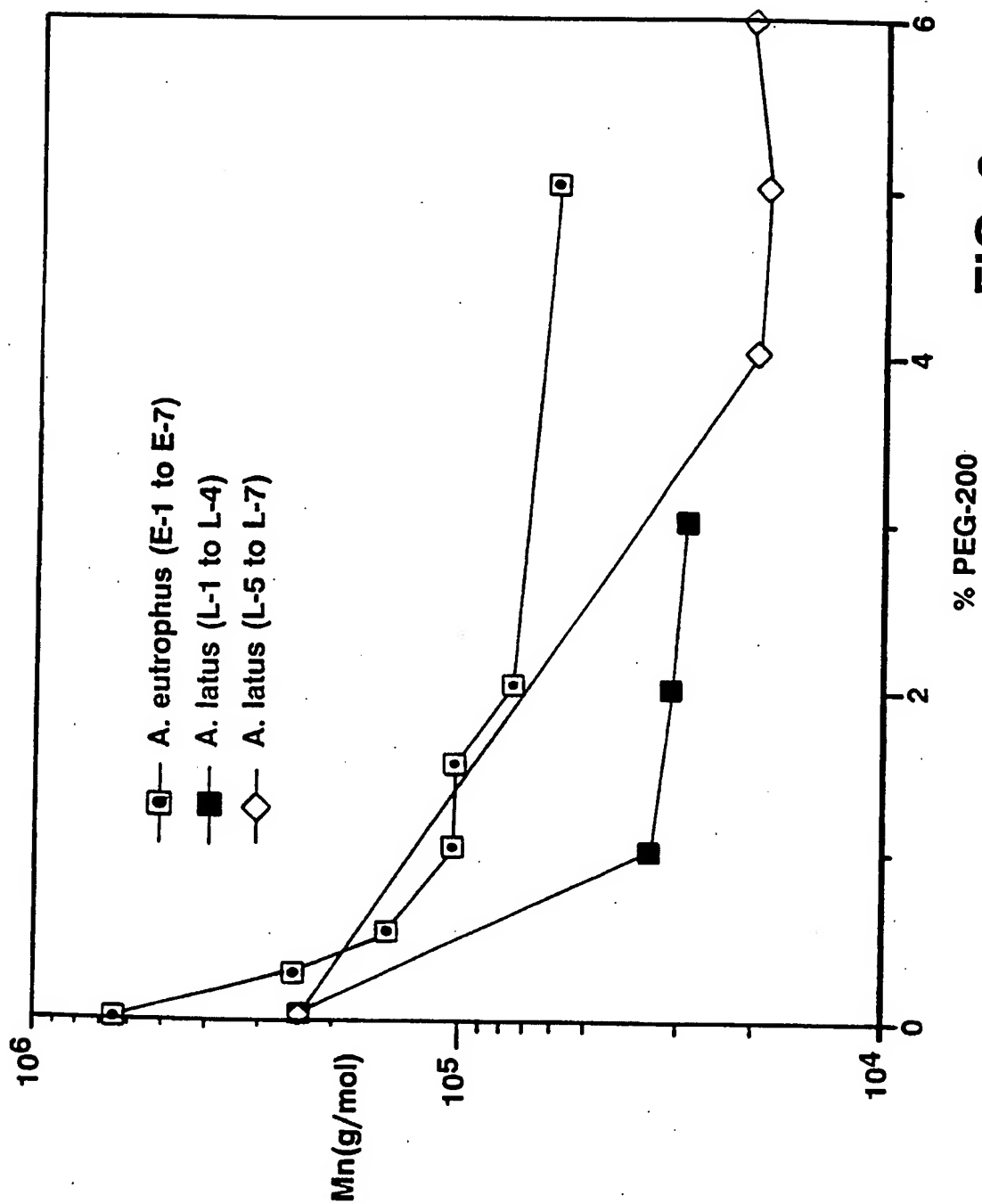FIG. 7C

-60   -40   -20   0   20

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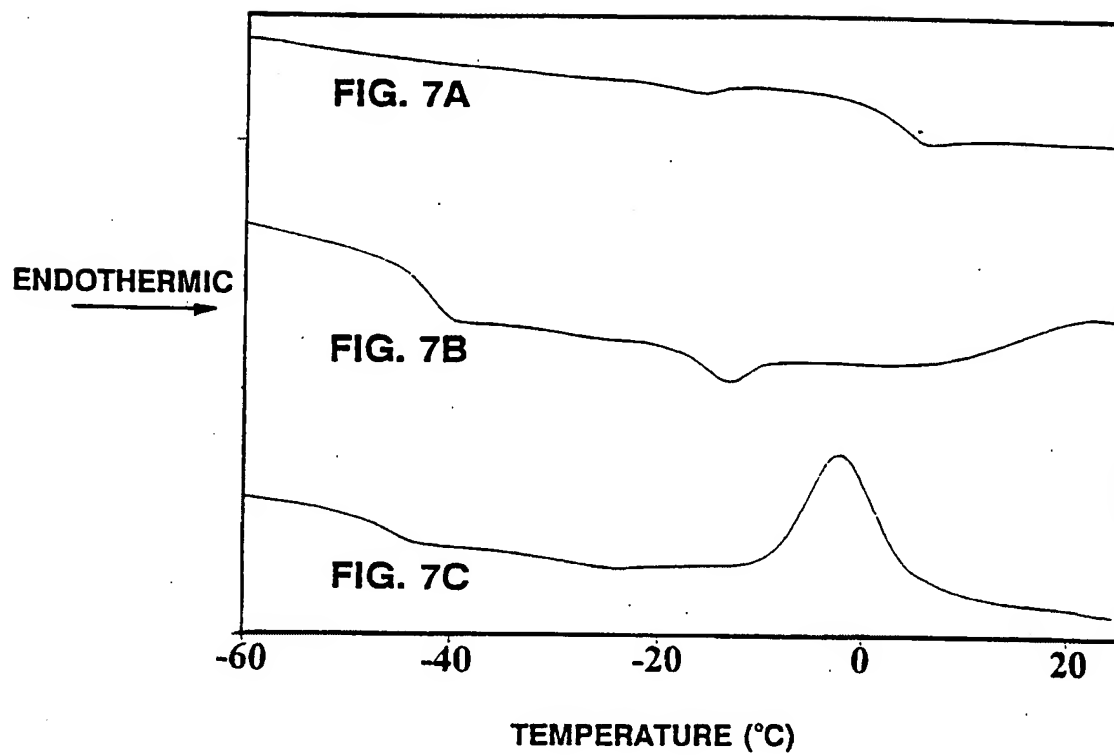
FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10396

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C08G 63/00; C12P 7/12, 7/62; C12N 1/12
US CL :435/135, 252.1, 280, 822, 829; 528/354, 361
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/135, 252.1, 280, 822, 829; 528/354, 361

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,477,654 (HOLMES ET AL) 16 October 1984, see entire disclosure.	1-30
A	US, A, 5,200,332 (YAMANE ET AL) 06 April 1993, see entire disclosure.	1-30
Y	US, A, 5,225,227 (YALPANI) 06 July 1993, see entire disclosure.	1-30
A	US, A, 5,264,546 (ANDERSON ET AL) 23 November 1993, see entire disclosure.	1-30
A	US, A, 5,344,769 (WITHOLT ET AL) 06 September 1994, see entire disclosure.	1-30
A	US, A, 5,395,919 (LEE ET AL) 07 March 1995, see entire disclosure.	1-30

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be part of particular relevance
"E"	earlier document published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 NOVEMBER 1995	**1 2 DEC 1995**

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5,434,062 (GROLEAU ET AL) 18 July 1995, see entire disclosure.	1-30
X --- Y	Chemical Abstracts, Volume 117, Number 24, 1995, abs no. 234736f Kleinke et al, "Melt polymerization of polyhydroxyalkanoates with compounds bearing at least two reactive groups, e.g., acid and/or hydroxyl groups" Abstract of EP 491,171 issued 24 June 1992, see entire disclosure.	26 --------- 1-25, 27-30

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